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(u) Polymerization of a Quinone-Crosslinked Marine Bioadhesive Protein

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Herbert Waite

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The marine adhesive proteins of some six different invertebrates have been isolated and partially sequenced. These proteins are characterized by having high isoelectric points and high levels of lysine and Dopa. There are few unifying features in the primary sequence. All of the proteins have repeat sequences with consensus peptides ranging from 4 to 10 amino acids. Most of these have C-terminal lysine and Dopa flanked on at least one side by glycine. The cross-linking of these proteins is mediated by catecholoxidase. This enzyme catalyzes the conversion of Dopa to o-quinone. The latter tautomerizes to α,β-dehydro-functional groups that are again oxidized by the enzyme, this time to α,β-dehydro Dopaquinone. The latter is expected to undergo a diverse assortment of nucleophilic addition reactions.

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PRINCIPAL INVESTIGATOR: J. Herbert Waite

CONTRACTOR: University of Delaware, College of Marine Studies

CONTRACT TITLE: Polymerization of a Quinone-Crosslinked Marine Bioadhesive Protein

START DATE: 1 September 1986

RESEARCH OBJECTIVE: The main objectives of this research are A. to survey the diversity of DOPA-containing marine adhesive proteins or cements, and B. to examine the chemical mechanism of quinone-tanning (i.e. cross-linking) of DOPA-containing proteins.

PROGRESS (Year 3): SEQUENCE STUDIES - The adhesive proteins of four organisms in addition to those from the original mussels, Mytilus edulis and M. californianus, have been characterized and partially sequenced. Consensus peptides from these proteins are shown in Table 1. Consensus peptides are those often repeated in the primary structure of the respective proteins but they are not necessarily the only sequences in the proteins. The three mussels Mytilus edulis, M. californianus and G. demissa all share the motif

Z-Dopa/Tyr-X-Y-Z-Dopa/Tyr-Lys

where Z is Ser at Thr in the two species of Mytilus and Gly in Geukensia. X and Y are usually 3- or 4-Hyp in Mytilus and variable in Geukensia, although Y is frequently pro/Hyp. The fourth mussel listed in Table 1, Aulocomya ater, from the southern coast of Chile, has a much shorter consensus sequence with only one DOPA per peptide seven amino acids long. The variable sixth position X is a hydrophobic amino acid. Lysine in position 7 is as often

hydroxylated (δ-hydroxylysine) as not. The reef-building polychaete secretes a composite marine cement that has a glycine-rich consensus peptide with 2 DOPAs (per nine residues) separated from one another by a single glycine. In the trematode Fasciola, Dopas are commonly separated from one another by 2 amino acids (Glys, Asp, Ser) and from C-terminal lysine by a single Gly. The second Fasciola protein sequenced is distinct from all others in containing no Lys, in its low MW, and in having the lowest pI. A consensus peptide, GlyHisGlyDopa, repeated perhaps 6-8 times is inferred from products of digestion by pepsin.

The diversity of DOPA-bearing sequences encountered thus far in naturally occurring proteins that undergo quinone-tanning suggest that this process is not restricted to a single monolithic sequence and that further unique and as of yet unknown functions may be served by the sequences of these proteins.

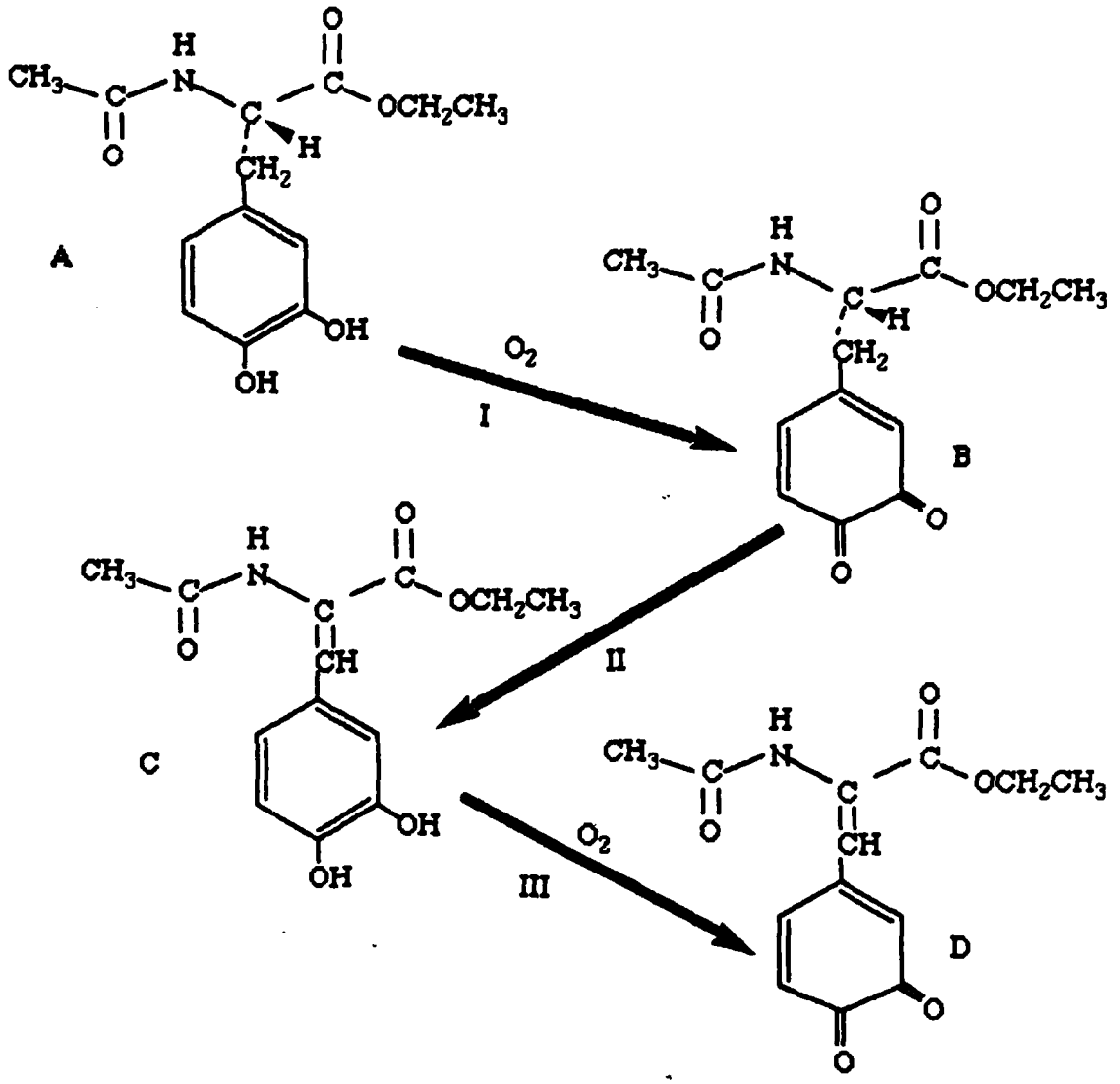
CROSS-LINKING STUDIES

In an attempt to elucidate the nature of the chemical species participating in curing of adhesive protein by covalent crosslinking, we have investigated the oxidation products of a synthetic peptide analogue, N-acetyl-DOPA ethyl ester (NAc-DEE), together with other related substances, under conditions felt to approximate the salinity and pH found in the native environment of the mussel Geukensia demissa. We have observed that the quinones derived from DOPA-containing molecules such as NAc-DEE undergo a novel oxidative rearrangement to form a catecholic enamic acid

derivative. This derivative, N-acetyl-α,β-dehydroDOPA ethyl ester, was characterized by taking advantage of the unique difference spectra between borate and HCl solutions of catechols bearing, α,β-unsaturated carbons. Any loss of the unsaturation would result in the formation of a tetrahedral carbon adjacent to the ring, with the concomitant loss of the characteristic spectra. Provided no de-acetylation or de-esterification had occurred, α,β-dehydroDOPA ethyl ester then has the probable structure shown in Fig. 1C.

The rearrangement of the quinone to form an α,β-desaturated DOPA derivative was probably spontaneous, for the following reasons. Firstly, the rearrangement occurred following enzymic oxidation utilizating either mushroom tyrosinase or byssal catechol oxidase, which suggests that the precise nature of the active site is irrelevant for the rearrangement. Secondly, the reaction was inhibited by ascorbate, which suggests that the quinone must first dissociate from the enzyme, where an enzyme is used for oxidation, before the rearrangement occurs. Thirdly, the rearrangement was effected by non-enzymic oxidizing agents.

The significance of these observations is potentially far-reaching. Our oxidation studies suggest that the yield of α,β-dehydroDOPA moieties from DOPA analogues can be quite high. Side reactions, such as the inter- and intramolecular Michael-type addition reactions occurring when DOPA or dopamine are oxidized, were avoided by using only N-acetylated DOPA analogues, (or, where appropriate, substrates lacking a free amine group). These data and preliminary observation on synthetic DOPA-containing decapeptides



A N-ACETYLDOPAETHYLESTER

B N-ACETYLDOPAQUINONE ETHYLESTER

C N-ACETYLTATSUIC ACID ETHYLESTER

D N-ACETYLQUINONYLTATSUIC ACID ETHYLESTER

I CATECHOLOXIDASE (CATECHOL:O_2 OXIDOREDUCTASE)

II TAUTOMERIZATION REARRANGEMENT

III CATECHOLOXIDASE

suggest that perhaps 50% of oxidized DOPA moieties (i.e. DOPA quinones) might undergo this rearrangement under appropriate conditions. Consequently, since the rearrangement appears to be spontaneous, oxidized DOPA residues in adhesive proteins may yield significant amounts of α,β-dehydroDOPA, or derived substances. Furthermore, HPLC analyses of hydrolysates of mussel byssus, which contains much DOPA protein, gave results consistent with the presence of large amounts of byssal non-DOPA catecholic moieties similar in nature to α,β-dehydroDOPA.

Although the presence of these novel oxidation products of DOPA residues in natural adhesive peptides has not yet been unambiguously confirmed, nevertheless, current evidence strongly suggests that they exist. Since enamines, which are very reactive molecules, participate in reactions quite different from those of quinones, the nature of the hardening process in bioadhesives containing o-quinonyl enamine residues may be radically different from that hitherto anticipated, and may have important consequences for the structure and properties of cured adhesive.

INVENTIONS - *one*

PUBLICATIONS

A. This program:

Waite, J. H. (1986) Mussel glue from Mytilus californianus: a comparative study. J. Comp. Physiol. B. 156, 491-496.

Mascolo, J. M. & Waite, J. H. (1986) Protein gradients in byssal threads of some marine bivalve molluscs. J. Exp. Zool. 240, 1-7.

Marumo, K. & Waite, J. H. (1987) Prolyl 4-hydroxylase in the foot of the marine mussel Mytilus edulis purification and characterization. J. Exp. Zool. 244, 365-374.

Waite, J. H. (1987) Nature's underwater adhesive specialist. Int. J. Adhes. and Adhesives 7, 9-15.

Waite, J. H. & Rice-Ficht, A.C. (1987) Presclerotized eggshell protein from the liver fluke Fasciola hepatica. Biochem. 26, 7819-7825.

Williams, T., Marumo, K., Waite, J. H. & Henken, R. (1988) Mussel glue has an open, extended conformation. ABB, in press.

Submitted Manuscripts

Waite, J. H. & Rice-Ficht,A. C. (1988) A histidine-rich protein from the vitellarial cells of the liver fluke, Fasciola hepatica. To: J. Biol. Chem.

Waite, J. H., Hansen, D. & Little, K. T. (1988) Adhesive protein of ribbed mussels: a natural glue with some features of collagen. To: J. Biol. Chem.

Rzepecki, L., Nagafuchi, T. and Waite, J. H. (1988) Oxidative synthesis of α,β-dehydroDOPA derivatives and their potential involvement in auto-tanning of DOPA-containing bioadhesive proteins. To: J. Biol. Chem.

Nagafuchi, T. and Waite, J. H. (1988) Adsorption of mussel adhesive protein to glass and polypropylene. To: Biomaterials.

TRAINING ACTIVITIES

Bernardo Estupiñan and Karen Long received their M.S. degrees in the summer of 1988. Thesis titles were: "Study of polyphenolic protein as inducing agent in the settlement of Mytilus edulis larvae", and "Hemolytic toxins of two marine jellyfish", respectively.

Tatsuhiko Nagafuchi has completed two years of postdoctoral research and is now assistant professor of orthopaedics at Jikei University, Tokyo.

Five students are currently working on this research: Douglas Hansen, Stefan Samulewicz, Luke Huggins, and Kathy Little. Postdoctoral fellow: Leszek Rzepecki.

Women/minorities - 1

noncitizen - 1 (Scotland)

Table 1. Summary of consensus sequences from various Dopa-containing proteins in nature. Symbols used for Dopa and hydroxyproline are YOH and POH, respectively.

Species	Mr(kD)	pI	Sequences	#Repeats
PHYLUM MOLLUSCA				
Mytilus edulis byssal adhesive	130	>10	AKP(OH)SY(OH)POHPOHTYOHK	>60
			AKPTY(OH)K	13
M. californianus byssal adhesive	85	>10	ISYOHPOHPOHTYOHKPOHK	15
Geukensia demissa byssal adhesive	130	8.2	QTGYOH X_1X_2GY(OH)K	>50
			A/TGYOHX_1X_2GY(OH)K	10
			X_1 = hydrophobic amino acid	
			X_2 = pro; hyp	
Aulocomya ater byssal adhesive	120	9.0	AGGYOHG X K(OH)	>20
			X = hydrophobic, branched chain	
PHYLUM ANNELIDA				
Phragmatopoma californica cement	35	8.4	X GGYOHGYOHGAK	>20
			X = hydrophobic, branch chain	
PHYLUM PLATYHELMINTHES				
Fasciola hepatica eggshell precursor	31	7.2	GGGYOHX_1X_2YOH G K	?
	10	6.6	GHGYOH	6
			X_1X_2 = Gly, Ser, Asp	